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Description of a Closed Plastic Bag System for the Collection and Cryopreservation of Leukapheresis-Derived Blood Mononuclear Leukocytes and CFUc from Human Donors

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Hemopoietic stem cells were collected from blood by means of continuous-flow centrifugation. The therapeutic use of large quantities of autologous blood stem cells requires a suitable, reliable and easily practicable cryopreservation technique which prevents loss of cell number and viability. This paper describes a closed plastic bag system consisting of one part for the collection and freezing of blood-derived mononuclear cells (MNC), among them granulocyte/macrophage progenitor cells (CFUc), and of a second part for thawing the cryopreserved cells and washing them free of DMSO before transfusion to a patient.

In a series of 20 leukaphereses, the average number of collected MNC and CFUc was about 11×10^9 and 8×10^5 respectively. The recovery rate of the leukapheresis derived MNC and CFUc after cryopreservation, thawing, and washing was demonstrated to be 90 per cent or better. Sequential leukaphereses in the same donor showed little effect on red and white blood cell concentration. However, there was a significant decrease in the donor's blood platelet concentration prior to the third leukapheresis.

IN RECENT YEARS, bone marrow transplantation has become important in the treatment of aplastic anemia and of certain types and stages of leukemia. The essential elements in bone marrow cell suspensions are hemopoietic stem cells capable of indefinite replication and differentiation along the pathways of the various hemopoietic cell renewal systems. It has been known for several years that pluripotent hemopoietic stem cells are present in the blood of mice,[12] dogs,[3] and man.[15] It is quite conceivable on the basis of the available data that hemopoietic stem cells migrate constantly through the blood stream either to differentiate or die and thus leave the blood stem cell pool, or to return to hemopoietic niches whenever there is a local demand for the restoration of hemopoietic activity.[9] Obtaining stem cells from the blood by means of a blood cell separator would have the distinct advantage over a bone marrow harvest, since such continuous-flow leukapheresis is generally well tolerated and can be performed repeatedly on an outpatient basis without the risks associated with anesthesia, or the discomfort following multiple bone marrow punctures.

In man it is not possible, as yet, to identify conclusively the presence of pluripotent hemopoietic stem cells in a blood mononuclear leukocyte suspension by means of morphological or cell culture techniques. However, it is possible to identify colony-forming units (CFUc) which are considered to represent granulocyte/macrophage progenitor cells.[2] It has been conclusively demonstrated in dogs that there is a direct correlation between the number of CFUc in a blood mononuclear leukocyte suspension, and its ability to repopulate a radiation-induced bone marrow aplasia.[7] It appears reasonable to use the determination of the number of CFUc in a suspension of mononuclear leukocytes as an indicator for the presence of pluripotent hemopoietic stem cells.

Received for publication December 18, 1978; accepted April 29, 1979.

Supported by the Deutsche Forschungsgemeinschaft, SFB 112.

0041-1132 80/0500/0293 $00.90 © J. B. Lippincott Co.

The retransfusion of cryopreserved autologous blood stem cells might be useful for early hematologic reconstitution following high-dose irradiation and/or chemotherapy. In order to utilize this therapeutic concept it appeared important to develop a reliable approach which would allow for the sterile collection and freezing of leukapheresis-derived mononuclear leukocytes including CFUc and would also be suitable for cell washing after thawing in preparation for retransfusion. It is the purpose of this paper to describe a closed plastic bag system for collection, storage and washing of mononuclear blood leukocytes from human donors, and to report on the efficiency of the cryopreservation procedure using the CFUc assay as a test system for stem cell viability and sterility.

Material and Methods

Twenty healthy, male volunteers (ages 21 to 30 years), who were regular blood and leukapheresis donors for cell support of patients in the university hospital, were subjected to continuous-flow leukapheresis. Each donor was fully informed of the risk involved in being placed on the cell separator for a period of four hours to obtain mononuclear leukocytes. Before commencement of leukapheresis each donor was given a thorough physical examination.

The blood leukocytes were procured by means of a continuous-flow blood cell separator.* Each individual run took four hours, processing a total of 12 liters of blood. The blood flow rate was 50 ml per minute and the centrifuge speed was adjusted to 800 rpm. The following components were added to the total blood volume processed: 170 IU heparin per kg body weight into the input line to maintain sufficient anticoagulation, 350 ml ACD to prevent platelet aggregation, and 900 ml plasmagel† to enhance the erythrocyte sedimentation. Instead of plasmagel which is not commercially available in the USA, hetastarch‡ can also be used without any effect on cell yield. The effectiveness of anticoagulation was repeatedly monitored by determination of the partial thromboplastin time, the thrombin time and fibrinogen. After discontinuation of leukapheresis, protamine was given.

It was the purpose of the leukapheresis procedure to collect an enriched mononuclear cell fraction from the buffy coat. To avoid cell clumping in the frozen and thawed leukapheresis-derived cell suspension, the contamination of polymorphonuclear cells (PMN) in the collected buffy coat cell suspension was kept as low as possible. To attain this, the red blood cell (RBC) interface in the centrifuge bowl was located about 1 mm outside the white blood cell (WBC) collection ports towards the periphery of the bowl (Fig. 1).

* American Instruments, Silver Spring, MD.
† Braun-Melsungen, West Germany
‡ McGaw Labs., Irvine, CA.

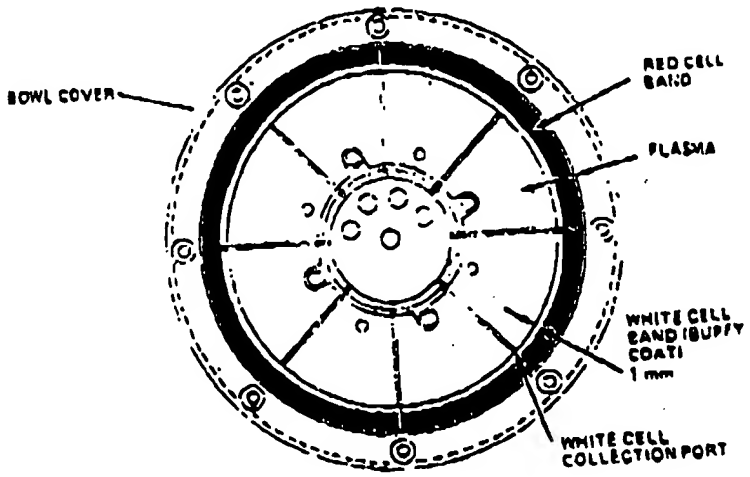


FIG. 1. White blood cell collection port location at centrifuge bowl cover for collection of mononuclear blood leukocytes.

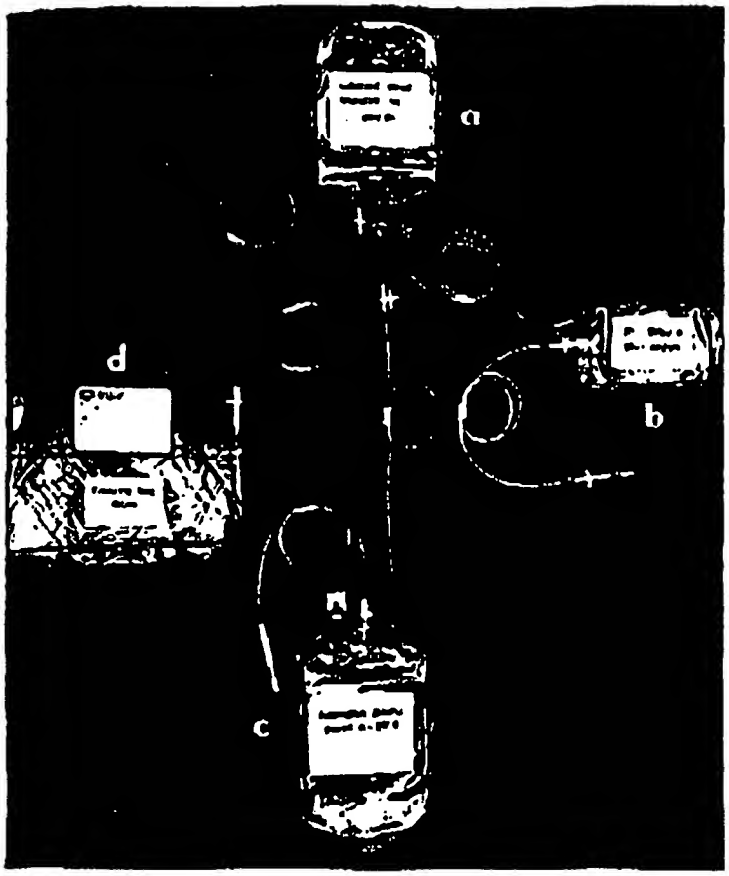


FIG. 2. Collection- and freezing-bag system. a. Collected blood leukocyte suspension. b. 20% DMSO in 90 ml medium. c. Autologous plasma, stored at -20C. d. Freezing bag, 180 ml.

The bag system developed consists of commercially available plastic bags§ of different sizes (150,600 and 1,000 ml) and of a freezing bag‖ (200 ml). The bags are connected to each other by integral plastic tubing as shown in Figures 2 and 3. To guarantee absolute sterility the bag systems were irradiated in toto using a dose of 3.5 Mrad from an electron linear accelerator. The leukocyte suspension derived from the blood cell separator was collected in a 600-ml plastic bag as part of the "collecting and freezing bag system" and then prepared for freezing (Fig. 2 and 4). After centrifugation of the whole bag system at 600 × *g* for 15 minutes, the supernatant plasma was transferred to a separate bag which was then sealed off and stored at −20 C until needed for the thawing process. To the leukocyte sediment (90 ml) the same volume of 20% DMSO in S-MEM (Minimum Essential Medium with Earle's salt without L-glutamine) was added so that the resulting total volume was 180 ml, and the final DMSO concentration was 10 per cent. The cell suspension was then transferred to the freezing bag which was sealed off for freezing.

The freezing bag was sandwiched between two copper plates (1-cm distance) and frozen in an automatically controlled freezer¶ at a cooling rate of 1 to 2 C per minute down to −40 C, then of 7 C per minute down to −130 C. The bag was stored for 24 hours in the gaseous phase of liquid nitrogen, then transferred into the liquid phase for prolonged preservation.

Thawing of the frozen cell suspension was performed within one minute in a waterbath at 52 C, in order to minimize exposure time of

§ Fenwal Labs., Deerfield, IL.

‖ Union Carbide, Chicago, IL.

¶ Cryosan, Midden Beemster, The Netherlands

hemopoietic stem cells to DMSO which was found to be stem cell toxic above 0 C.[11] Immediately afterwards the freezing bag as well as the bag containing the thawed plasma were connected to the "washing bag system" for mixing both fluids (Fig. 3 and 4). After centrifugation of the whole bag system at 600 × g at 10 C for 15 minutes, the supernatant fluid was transferred to the waste bag. The cell sediment was resuspended in about 500 ml S-MEM. This procedure was repeated once to eliminate almost all DMSO. The final cell sediment was made ready for transfusion into the recipient by resuspending in about 50 ml S-MEM.

For determination of the quantity and viability of blood stem cells, heparinized venous blood samples and leukapheresis-derived leukocytes were separated over a Ficoll-Isopaque gradient. Mononuclear cells (MNC) were collected at the interface, washed twice, and resuspended in culture medium which was prepared as follows: 80 ml S-MEM were mixed with 20 ml fetal calf serum and supplemented with 0.4 ml MEM vitamins,** 1.0 ml of 100 mM TC sodium pyruvate,** 0.04 ml of a 42-mg per ml solution of DL-serine,** 1.4 ml of 200 mM L-glutamine,** 0.16 ml of a 10-mg per ml solution of L-asparagine,‡‡ 0.8 ml of MEM amino acids solution-50×,** 0.4 ml of nonessential amino acids solution-100×,** 8,000 IU of penicillin, and 8 mg of streptomycin dissolved in 0.8 ml S-MEM. The cryopreserved and thawed cell suspension was prepared for plating without Ficoll-Isopaque gradient centrifugation.

For the measurement of colony-forming units (CFUc), a modification of the soft agar culture technique was employed.[5] The cell suspension, containing 0.3% agar, was mixed in 35-mm Petri dishes with colony-stimulating activity, using human placental conditioned medium (15% v/v) as described by Burgess *et al.*[5] The culture dishes were incubated for 10 days in a 100 per cent humidified atmosphere at 37 C with 5% CO_2.

Cultures were set up in triplicate at three

** Gibco, Grand Island, NY.

‡‡ Microbiological Assoc., Walkersville, MD.

** Serva, West Germany

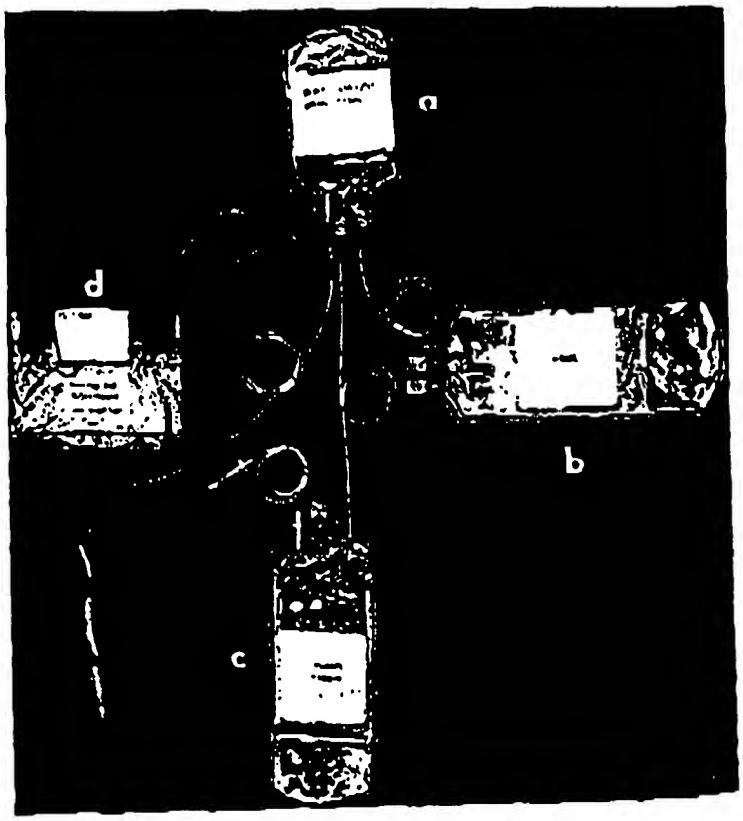
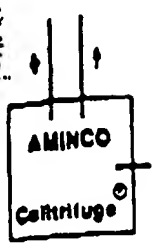


FIG. 3. Thawing- and washing-bag system. a. Thawed autologous plasma. b. Waste. c. Medium, 1,000 ml. d. Freezing bag, 180 ml thawed cell suspension.



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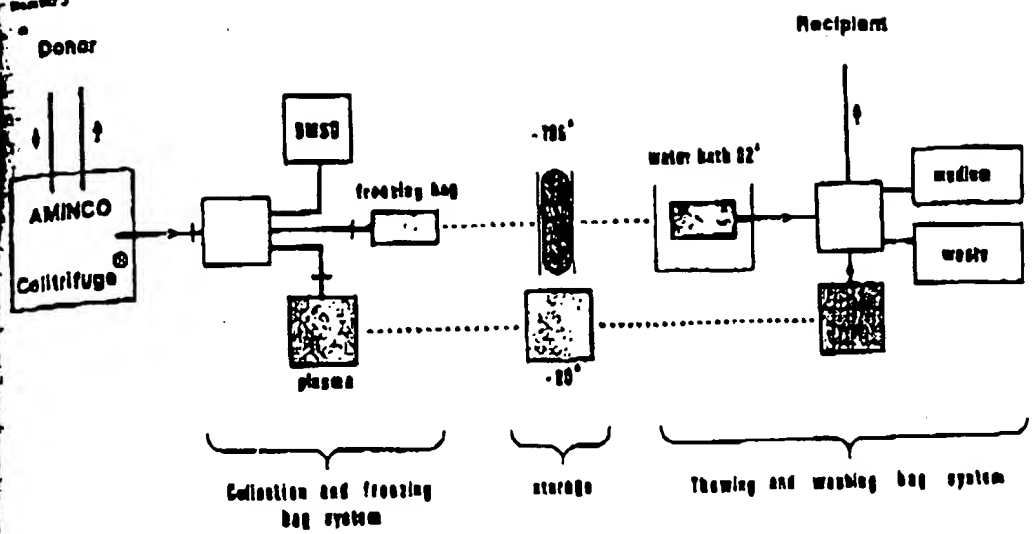

FIG. 4. Methodological approach of cryopreservation and preparation of large quantities of leukapheresis-derived blood leukocytes for transfusion into recipients.

different cell concentrations (0.25, 0.5 and 1 × 10^6 MNC per dish). Colonies, defined as aggregates with more than 50 cells, were counted using a stereo-microscope at 50×. For morphological classification, colonies were picked up with a modified Pasteur pipette and placed on microscope slides, stained with 0.05% acridine orange dissolved in tissue culture medium, and examined at 400× magnification with ultraviolet light.

Results

Table 1 indicates the efficiency of the method for cryopreservation of blood stem cells. The number of MNC and CFUc before and after cryopreservation are the mean values obtained from 20 individual leukapheresis. The mean number of MNC and CFUc collected per run was 11.3 × 10^9 and 7.5 × 10^5, respectively. The corresponding values after freezing, thawing, and washing were 10.0 × 10^9 and 6.9 × 10^5. The mean recovery of MNC was 89.7 per cent and that of CFUc, 95.7 per cent. The percentage of granulocytes contaminating the collected leukocytes was about 10 per cent. Clumping of cells during the DMSO washout procedure was not observed for at least one hour after thawing. The mean storage time was 16.4 days. In the original cell suspension and in the cryopreserved, thawed, and washed cell suspension, colonies were identified morphologically. About 80 per cent of the cells could be recognized as differentiating neutrophils, including mature polymorphonuclear leukocytes. Colonies regularly contained more than 50 cells in both native and processed cell suspensions.

Seven different donors underwent three sequential four-hour leukaphereses on days 0, 2, and 4. The procedures were well tolerated without any exception. Taking the mean number of blood cells per µl just before onset of the first leukapheresis as 100 per cent, no significant change in the mean MNC, PMN and RBC concentration was

Table 1. *Yield of MNC and CFUc from 20 Leukapheresis Procedures*

	Before Cryopreservation		After Cryopreservation		% recovery	
	MNC × 10^9	CFUc × 10^5	MNC × 10^9	CFUc × 10^5	MNC	CFUc
Mean	11.3	7.5	10.0	6.9	89.7	95.7
SD	±2.7	±4.4	±2.2	±3.6	±10.8	±18.2
Range	5–15	1–14	4–14	1–13	75–112	67–132

Mean storage time (days): 16.4 ± 6.0 SD

measured prior to each subsequent leukapheresis. A significant decrease in the mean platelet concentration of 36.5 per cent ($p < 0.005$) was observed before onset of the third leukapheresis (Table 2).

Discussion

Cavins *et al.*[5] described the restoration of normal hemopoietic function in lethally irradiated dogs by means of autologous transfusion of cryopreserved hemopoietic blood stem cells. These early observations were extended by systematic preclinical studies in our group. In the autologous situation, transfusion of leukapheresis-derived and cryopreserved blood MNC into lethally irradiated dogs resulted regularly in a complete and permanent restoration of hemopoiesis.[1,4-8]

We have been able to obtain and cryopreserve large quantities of human MNC. The included blood CFUc appear to represent pluripotent hemopoietic stem cells which could be used in the treatment of hemopoietic failure in man. This concept has been applied for the reversal of blast-cell crisis in patients suffering from chronic myelocytic leukemia.[10] The number of cryopreserved, thawed, and washed CFUc that should be present in a blood leukocyte suspension to guarantee complete and permanent regeneration of an aplastic bone marrow lies between 20,000 and 40,000 per kg body weight. Therefore, sufficient MNC and CFUc cell doses for expected engraftment of a 70-kg adult can be obtained from two to three leukaphereses. Under these conditions, resultant cell collection from each leukapheresis can be frozen, stored, thawed and administered separately one bag after the other without delay.

As described elsewhere,[11] a single four-hour continuous-flow leukapheresis does not significantly affect the donor's blood cell concentration, which includes MNC, CFUc, PMN and RBC. The mean platelet concentration, however, decreased significantly by 28 per cent (on the average) in 35 single runs. Furthermore, we could demonstrate that the impact of three sequential leukaphereses on the same donor is little. Nevertheless, the donor's blood platelet concentration which never was seen below 100,000 per μl, must be considered as a limiting factor for leukapheresis and must be checked carefully.

Studies are under way to find out possible ways of increasing the output of CFUc per leukapheresis. In man, after high-dose pulse chemotherapy a rapid and greatly overshooting expansion of the peripheral blood CFUc pool occurs between the sixth and fourteenth day.[14] In the dog it was shown that the administration of dextran sulfate (15 mg per kg body weight) causes a tenfold increase in blood CFUc concentration.[16] Zander *et al.*[17] observed similar effects on blood CFUc concentration after pyran administration in mice and dogs.

Table 2. *Platelet Response to Three Successive Leukapheresis Procedures*

Donor	First	Second		Third	
	platelets ($\times 10^3/\mu l$)	plat. ($\times 10^3/\mu l$)	% change*	plat. ($\times 10^3/\mu l$)	% change
H.Sch.	224.0	263.0	+17.4	186.0	−17.0
H.O.	334.0	223.0	−33.2	205.0	−38.6
R.K.	225.0	110.0	−51.1	150.0	−33.3
H.G.	302.5	157.5	−47.9	167.5	−44.6
T.E.	270.0	317.5	+17.6	182.5	−32.4
R.St.	426.0	310.0	−27.1	217.5	−48.8
G.W.	327.5	170.0	−48.1	196.0	−40.5
mean	301.1	221.6	−24.5	186.2	−38.5
SD	±70.5	±79.5	±30.1	±22.7	±10.4

* Count obtained before start of first procedure = 100%

The length of storage time appears to be of no consequence for the recovery and viability of the thawed CFUc, provided that the cells are stored in the liquid phase of nitrogen at −196 C.[8] These experiences are in contrast to those of Appelbaum *et al*.[1] who found a relationship between storage time and engrafting ability of transfused canine marrow cells. However, the cells in their studies were cryopreserved in the vapor phase of liquid nitrogen.

The present results indicate that there is only a small loss in the number of cells or in cell viability after cryopreservation, thawing, and washing of leukapheresis-derived human MNC. High recovery of both MNC and CFUc after cryopreservation can be attributed to collection of a granulocyte-poor leukocyte suspension, which avoids cell-clumping as often observed in frozen and thawed blood leukocyte suspensions during the DMSO wash-out procedure.

The data presented provide evidence that the collection and cryopreservation of significant stem cell numbers from the blood is feasible and practical under blood banking conditions. Particularly in the autologous situation, the transfusion of stored viable hemopoietic stem cells might allow intensification of chemotherapy in patients with solid tumors or prolongation of the remission phase in patients with chronic myelocytic leukemia after cytotoxic treatment. The possible therapeutic use of stored blood stem cells is dependent on their collection by simple means without risk to the donor and on their efficient cryopreservation without loss of cell number and viability. Stem cell collection is possible with a blood cell separator and can be done like any other blood donation procedure on a continuous-flow centrifuge. It is envisaged that blood stem cell transfusion may well become in the future a firmly established method of blood banking.

Acknowledgment

The authors thank Miss G. Baur and Miss C. Ritter for their expert technical assistance and Mr. M. Rudolf from the Dept. Verfahrenstechnik, Bundesforschungsanstalt für Ernährung, Karlsruhe, for sterilizing the bag systems.

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